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# Identifying Genetic Linkage Groups and Markers for Leaf Miner Resistance in Chickpea through QTL Analysis and Field Validation

Lina Ali [1], Mustapha El Bouhssini [2], Tawffiq Istanbuli [3], Muhammad Imtiaz [4], Alsamman M. Alsamman [5], Ahmed E. Nassar [6], Michael Baum [7], Aladdin Hamwieh [6]

[1] Department of Plant Protection, Faculty of Agriculture, University of Aleppo, Syria.
[2] Affiliation African Genome Center, Mohammed VI Polytechnic University, Ben Guerir, Morocco.
[3] International Center for Agricultural Research in the Dry Areas (ICARDA), Terbol, Lebanon.
[4] HarvestPlus, Pakistan Office, Crop Sciences Institute, NARC, Park Road, Islamabad 44000, Pakistan.
[5] Department of Genome Mapping, Agricultural Genetic Engineering Research Institute, Agricultural Research Center, Giza P.O. Box 12619, Egypt.
[6] International Center for Agricultural Research in the Dry Areas (ICARDA), Giza, Egypt.
[7] International Center for Agricultural Research in the Dry Areas (ICARDA), Rabat, Morocco.

* **To whom correspondence should be addressed: a.hamwieh@cgiar.org**

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## Abstract

Chickpea is a commonly grown crop, but it is vulnerable to biotic and abiotic stresses. Leaf miner (*Liriomyza cicerina*) is a pest that can cause severe yield losses of up to 40% if not properly controlled. This study was conducted at ICARDA (Aleppo, Syria) during the 20112012 growing seasons. Two recombinant inbred lines, ILC 5901 (LM resistant) and ILC 3397 (LM susceptible), were crossed to yield 350 $F_2$ plants, which were then screened for pathogen tolerance. The resistance of the plants was screened using a scale of one to nine, with 1 indicating complete resistance and 9 indicating complete susceptibility. A set of 600 simple sequence repeat (SSR) markers were validated on both parents, and 51 of these markers showed variation and were used to construct a genetic linkage map. QTL analysis was performed to determine the linkage groups responsible for line variations. The QTL analysis found that linkage groups TA37, TA34, and H4F03 were responsible for 22% of line variations, while unmapped NCPRG48 and H1C092 revealed 55.3% and 26.8% of the LIS variance, respectively, and displayed a warped dominance toward the susceptible parent. The H1C092 marker, which is significantly associated with LM, is located on Chr3 near a gene encoding the glutathione S-transferase gene family enzyme, which protects cellular macromolecules from attack by reactive electrophiles. The highly associated markers were field tested for three years to confirm their connection with LM resistance in 200 chickpea genotypes. The study showed marker-associated selection, which could accelerate the conventional breeding of LM-resistant chickpea germplasm. The markers linked to LM resistance and the identification of the protective enzyme gene offer promising avenues for further research. This study represents a significant step forward in understanding the genetics of LM resistance in chickpea and provides valuable information for breeding programs aimed at improving chickpea production.

**Keywords:** leaf miner, chickpea, SSR markers, *Liriomyza cicerina*

## Introduction

Chickpea (Cicer arietinum L.), or Bengal gram, is one of the seven Neolithic essential crops of the Near Eastern Fertile Crescent [1]. It is the first legume food source in South Asia and the third globally, after common bean and field pea [1]. Additionally, it is a significant crop in the Middle East, Mediterranean, India, and Ethiopia, providing both food and protein [2; 3]. It is grown in over fifty countries, including North Africa, the Indian subcontinent, the Middle East, southern Europe, the Americas, and Australia [1]. With an annual production of 17.2 million tons, chickpeas are grown on 17.8 million ha of land worldwide [4; 5]. Chickpeas are classified into two species: Desi and Kabuli. Kabuli (macrosperma) has white flowers, little anthocyanin pigmentation, and beige seeds in the form of a ram's head, whereas Desi (microsperma) has pink flowers, anthocyanin pigmentation on stems, and a colored, thick seed coat [6]. Despite all of the chickpea's benefits, there have been some biotic and abiotic stresses that have reduced and threatened its annual production. [7; 8].

Among the biotic factors that pose a great danger to chickpea production is *Liriomyza cicerina*. Depending on the severity of the pathogen, leaf miner infection could result in yield losses of up to 36% in West Asia, North Africa, and Southern Europe [9]. According to ICARDA, chickpea yield losses due to leaf miner infection could represent up to 40% of total production in Syria and other countries [10; 9]. Insecticides are the traditional method of LM control; however, this method has many drawbacks, including increased pest resistance to chemicals, which may have negative effects on human health and cause catastrophic diseases such as cancer [11]. Therefore, there is an urgent need to develop nontraditional methods of controlling this pest. Host plant resistance, also known as the premeditated use of resistant breeds to reduce the harmful effects of pests, is the most effective, sustainable, and safe way of maintaining crop production systems [12; 13; 14]. These resistant genotypes frequently suffer lower leaves damage compared with susceptible cultivars. Screening of chickpea germplasm was found to have useful levels of resistance to *Liriomyza cicerina* [15]. These resistant genotypes usually have less leaf damage than susceptible cultivars, which could be due to the organic acids in resistant germplasm [16]. The organic acids exuded by cicer species vary, but the most prevalent are malic, oxalic, succinic, citric, and quinic acids [17; 18].

Molecular genetics is an important method for selecting and developing resistant genotypes in a variety of species [19]. The detection of molecular markers that are closely linked to resistance genes is extremely beneficial in replacing time-consuming and frequently unreliable field evaluations with molecular techniques [20]. QTL is a term that describes the locations, numbers, amounts of phenotypic effects, and mechanisms of gene activity of breed factors that contribute to the inheritance of variable traits [21]. The QTL analysis uses a linkage map as a "framework" to pinpoint the chromosomal locations of genes conferring quantitative resistance [22]. Although it is broadly useful to study in many fields, QTL mapping is particularly important in agriculture. It is an accurate measure of yield quality and productivity in agricultural fields that serves as the culmination of an organism's life cycle [21]. Although RFLP (restriction fragment length polymorphism) is the most commonly used marker in QTL mapping, the current study employed SSR markers in linkage mapping [23]. The current study is a step forward in identifying marker-associated selection that could improve and speed traditional farming by developing novel resistant varieties for leaf miners.

## Materials and Methods

### The field experiment and disease score

The 350 tested $F_2$ were produced by crossing chickpea genotypes ILC5901 (LM resistance, multi-pinnate leaf type, small leaflet size) and ILC3397 (LM susceptibility, normal leaf type, large leaflet size) (Figure 1). They were planted on the ICARDA (International Center for Agricultural Research in the Dry Areas) field in Tel Hadya, Syria. The plants were graded on a 19 leaf infection scale (LIS) according to the percentage of infected

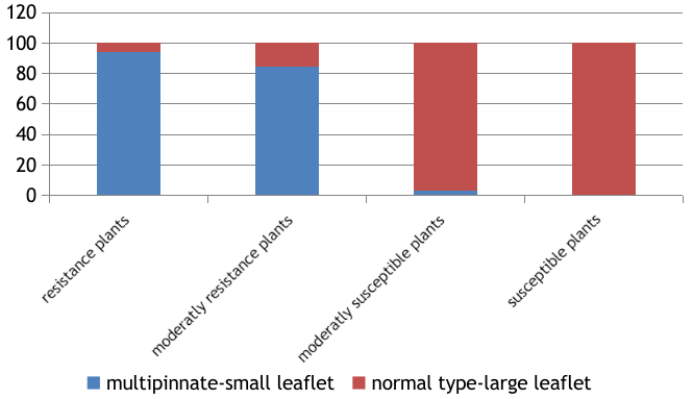


**Figure 1.** The two leaves type: A-first parent ILC3397, LM susceptible, normal leave type, large leaflets size. B-second parent ILC5901, LM resistance, multipinnate leave type, small leaflets size.

leaflets per plant, where 1 = no infections (no mines), 2= less than 5% and no defoliation, 3= less than 20% and no defoliation, 4= between 21% to 30% and no defoliation), 5= between 31% to 40% with little defoliation, 6= 41% to 50% and 10% of the lowest leaves were dropped, 7= 51% to 70% and 10% to 20% of the lower and upper affected leaves were defoliated, 8= 71% to 90% and 20% to 30% of diseased upper and lower leaves are lost, and 9 = miners observed on all the leaflets and falling more than 30% of the leaves. In general, plants with LIS=1-3 were considered resistant, LIS=4 plants moderately resistant, LIS=5-6 plants moderately susceptible and LIS=7-9 plants were susceptible [9]. The genotypes were screened when the susceptible parents showed LIS>5 to LM under natural insect infestations. The segregation of the leaf size (large or small leaflets) and type (multi-pinnate-small leaflet or normal type-large leaflet) were also collected from the field.

### DNA isolation

DNA was isolated from seedling leaves after 4-6 weeks using the cetyltrimethylammonium bromide (CTAB) technique, as follows: Fresh seedling leaflets were freeze-dried for three days. In an Eppendorf tube, two metal balls (4 mm in diameter) were ground into powder. The CTAB buffer solution has the following ingredients: 2% cetyl trimethylammonium bromide, 1% polyvinylpyrrolidone, 100 mM Tris-HCl, 1.4 M NaCl, and 20 mM EDTA. After mixing and vortexing the suspension, 1 ml of phenol/chloroform/isoamyl alcohol (25:24:1) was injected, and the tube was placed in a 60řC water bath for 60 minutes. After centrifuging the homogenate for 20 minutes (4000 RPM at 4řC), the aqueous upper phase was transferred to a new tube, and the process was repeated until the upper phase was definite. This solution will then be given 700 uL of isopropanol. The sample was centrifuged at 14,000 x g for 10 minutes before being washed twice using a washing buffer composed of 75% ethanol and 200 mM sodium. The supernatant was dried for 1020 minutes to eliminate residual ethanol before being dissolved in

100 ţL TE buffer (10 mM Tris HCl, pH 8, 1 mM EDTA). The chickpea primer sequences were retrieved from these articles [24; 25; 26; 26; 27; 28; 29] in order to use polymerase chain reaction. The thermocycler (Perkin Elmer PCR System 9700) was used to conduct PCR (10 ml mixture) with 25 ng template DNA, 10 pcr buffer, 5 pmol forward and reverse primers, dNTP, and 5 u of Taq DNA polymerase. The PCR technique divides the PCR analysis into three major steps: the first is denaturation, which requires 35 cycles of heated solution at 94 řC for 15 seconds; the second is annealing, which lasts 30 seconds at 72 řC; and the third is extension, which takes 5 minutes at 72 řC. The final step is PCR gel electrophoresis, which involves electrophoresizing the PCR products on an 8% polyacrylamide gel containing ethidium bromide.

### Statistical analysis

The JoinMap 4 software, which is used for genetic linkage calculations in experimental populations of diploid species, was used to construct genetic linkage mapping [30]. It improved the possibility of mapping genes for the enhancement of the chickpea crop by providing more knowledge of chromosome organization, parental relationships, and gene ordering [31]. The linkage groups were given to the chromosomes depending on recombination frequencies less than 0.45 (Θ >.45) and LOD greater than 3. MapQTL 6 software was employed for QTL mapping, as well as the genetic distances (centimorgans, cM) were determined using the Kosambi function [32; 30]. Utilizing composite interval mapping, it was observed that the significant QTL had a LOD threshold greater than 2 [33].

## Validation of associated PCR markers

To validate their association with leaf minor resistance and to investigate their potential linked function, five associated LM SSR markers (H1C092, H4F03, TA34, NCPGR48, and TA37). These markers have a significant association with the leaf minor phenotype in QTL analysis. The FIGS (Focused Identification of Germplasm Strategy) was used to pick 200 chickpea genotypes. It is a technique that has been scientifically proved to help crop breeding programs uncover important features in plant genetics more correctly and effectively, overcoming the disadvantages of more traditional approaches that are essentially hit-or-miss [34]. FIGS is a trait-based approach that helps genebank managers identify desired genetic material that is likely to have the desired trait [35]. Over three years (2014, 2015, and 2016), these genotypes were evaluated for LM resistance throughout three replicates each. The extracted DNA was used in PCR analysis for potential LM associated SSR markers using the aforementioned methods. The PowerMarker software [36] was used to examine the marker-trait relationship between genetic polymorphism in potentially associated SSR markers and LM resistance phenotypic variation. Using KASPspoon software [37], an *in silico* analysis and comprehensive investigation was conducted for the potential location of these PCR markers on the chickpea genome [38] and nearby genes.

## Results

### Phenotyping data

The results indicated only 35 (10%) out of 350 $F_2$ plants were resistant (LIS =<3), 52 plants (14.9%) were moderately resistant (LIS=4), 121 plants (34.6%) were moderately susceptible (LIS =5, 6), and 142 plants (40.7%) showed susceptible (LIS =7-9; Figure 2). $F_2$ plants showed about 94.3% of resistant plants (33 out of 35 plants) were multi-pinnate type leaf and small leaflet, and 99.29 % of susceptible plants (142 out of 143 plants) were normal type leaf and large leaflet (Table 1). A significant negative relationship (R= -0.92 and P < 0.001) discovered among leaf minor resistance and leaf type, and leaf size, while a high positive correlation was found between leaf type and leaf size (R=1; P < 0.001).


**Figure 2.** Frequency distribution of LM resistance of the 350 F2 plants derived from P1= ILC5901 (LM resistance) and P2=ILC3397 (LM susceptible). Values of the parents are shown by arrows.

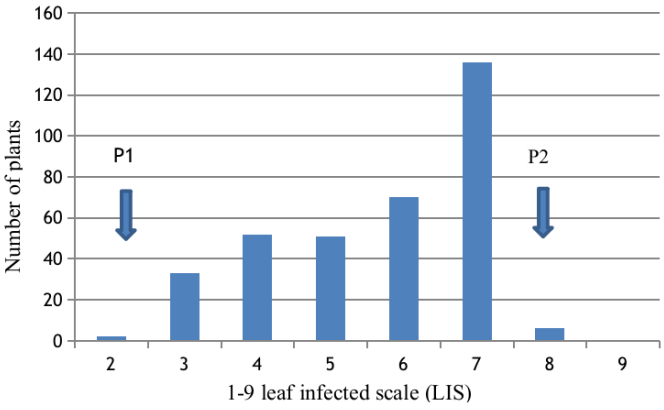


**Figure 3.** The distribution of leave type and leaflet size in 350 F2 plants.

### Mapping and QTL analyses

Validating 600 SSR markers on the parents (ILC5901 and ILC3397), it was determined that only 51 (8.5%) had polymorphisms, which were subsequently utilized for mapping. There were only 44 markers mapped on 8 linkage groups spanning 966.3 cM and seven (13.7 %) remained unlinked (GAA47, NCPRG48, TA106, H1B13, H1B09, TA140 and H1C092), with an average

**Table 1.** Percentage and the number of plants with the leave types and the size of leaflets of 350 F2 plants.

| Degree of resistance | Multipinnate-small leaflet | Normal type-large |
|---|---|---|
| leaflet | | |
| Resistance plants | 94.3 % (33 plants) | 5.7 % (2 plants) |
| Moderately resistance plants | 84.6 % (44 plants) | 15.38 % (8 plants) |
| Moderately susceptible plants | 3.3 % (14 plants) | 96.69 % (107 plants) |
| Susceptible plants | 0.7 % (1 plant) | 99.29 % (142 plants) |

**Table 2.** Percentage and the number of plants with the leave types and the size of leaflet of 350 F2 plants.

| S/n | Locus | a | h | b | - | $X^2$ | Significance |
|---|---|---|---|---|---|---|---|
| 1 | NCPRG48 | 19 | 33 | 42 | 2 | 19.6 | ******* |
| 2 | Gaa47 | 18 | 67 | 10 | 1 | 17.36 | ****** |
| 3 | H3F09 | 26 | 22 | 6 | 42 | 16.67 | ****** |
| 4 | H1B13 | 25 | 61 | 8 | 2 | 14.49 | ***** |
| 5 | H3G06 | 13 | 63 | 14 | 6 | 14.42 | ***** |
| 6 | TA144 | 37 | 48 | 11 | 0 | 14.08 | ***** |
| 7 | TS54 | 38 | 37 | 18 | 3 | 12.48 | **** |
| 8 | ta180 | 14 | 65 | 15 | 2 | 13.81 | **** |
| 9 | ncprg89 | 8 | 56 | 27 | 5 | 12.78 | **** |
| 10 | H3C-06 | 38 | 38 | 20 | 0 | 10.92 | **** |
| 11 | H4F03 | 32 | 31 | 32 | 1 | 11.46 | **** |
| 12 | H3D05 | 17 | 40 | 39 | 0 | 12.75 | **** |
| 13 | TS82 | 22 | 26 | 32 | 16 | 12.3 | **** |
| 14 | H3E04 | 34 | 32 | 26 | 4 | 9.91 | *** |
| 15 | TA1 | 12 | 52 | 31 | 1 | 8.45 | ** |
| 16 | H5D02 | 34 | 40 | 20 | 2 | 6.26 | ** |
| 17 | H4H08 | 33 | 36 | 26 | 1 | 6.6 | ** |
| 18 | H5F021 | 34 | 36 | 23 | 3 | 7.34 | ** |
| 19 | H1B09 | 17 | 40 | 35 | 4 | 8.61 | ** |
| 20 | TA140 | 27 | 42 | 9 | 18 | 8.77 | ** |
| 21 | H1D221 | 34 | 34 | 22 | 6 | 8.58 | ** |
| 22 | H4B08 | 20 | 37 | 33 | 6 | 6.6 | ** |
| 23 | H3A10 | 28 | 50 | 14 | 4 | 4.96 | * |
| 24 | H1F21 | 34 | 40 | 21 | 1 | 5.93 | * |

distance among the markers of 18.94 cM. Out of the 24 (47%) distorted markers in the 1:2:1 Mendelian ratio, the Chi-square testing indicated that 19 (37.2%) were distorted at a p-value <0.01, and five were highly distorted at a p-value <0.001 (Table 1).

QTL analysis identified that three linkage groups (LG2 (TA37), LG3 (TA34), and LG5 (H4F03)) explain 22% of the LIS variations, and they also confer chickpea resistance to a great extent. These linkage groups have flanking markers of (TS 54, TA200), (TA 125, H5H032), and (TR 29, H2I10). Interestingly, two unmapped markers (NCPRG48 and H1C092) were substantially associated with LIS, accounting for 55.3% and 26.8% percent

| No. | LG | Position | Locus | LOD | % Expl. | Additive | Dominance |
|---|---|---|---|---|---|---|---|
| 1 | UG | 3 | NCPRG48 | 16.77 | 55.3 | 1.50 | 1.37 |
| 2 | UG | 11 | 1C092 | 6.5 | 26.8 | 1.48 | 0.33 |
| 3 | 4 | 80. 6 | H4F03 | 5.23 | 22.2 | -0.83 | 1.31 |
| 4 | 1 | 167.89 | TA37 | 3.58 | 15.8 | -0.97 | -1.04 |
| 5 | 3 | 23.04 | TA34 | 2.24 | 10.2 | 0.86 | -0.37 |

**Table 3.** the marker associated with LM resistance in chickpea in addition to the additive and dominance effects across linkage groups (LG) and un-linked group (UG)

of the LIS variation, respectively (Table 2). These indicators revealed a dominant tendency toward the vulnerable parent. According to the additive effect for the markers with LOD >2, QTLs (NCPRG 48, H1C092, and TA34) source the susceptible chickpea genotype "ILC3397," while (TA37 and H4F03) source the resistant chickpea genotype "ILC5901". The QTLs (NCPRG48, H1C092, H4F03 and TA37) are major markers with a high explanation percentage (Table 3 and Figure 4).

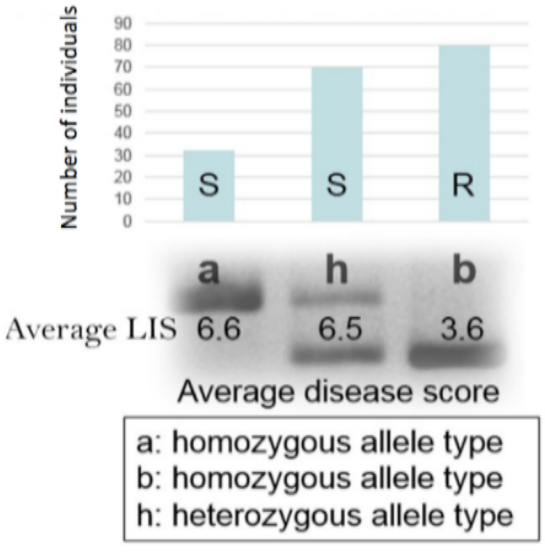


**Figure 4.** Statistical explanation and genetic state of the SSR marker "NCPRG48" in LM sensitive and resistant genotypes.

| Primer | PCR band size | year | pvalue |
|---|---|---|---|
| H1C092 | 375 | 2014 | $7.50 \times 10^{-03}$ |
| | | 2015 | $2.35 \times 10^{-02}$ |
| | 380 | 2014 | $4.61 \times 10^{-02}$ |
| | | 2015 | $2.18 \times 10^{-04}$ |
| H4F03 | 305 | 2016 | $3.72 \times 10^{-04}$ |
| NCPGR48 | 215 | 2015 | $1.09 \times 10^{-02}$ |
| TA34 | 280 | 2014 | $9.36 \times 10^{-04}$ |
| TA37 | 375 | 2015 | $3.27 \times 10^{-02}$ |

**Table 4.** Percentage and the number of plants with the leave types and the size of leaflet of 350 F2 plants.

## Validation of leaf miner associated QTLs

The highly associated markers were chosen for further experimental validation to confirm their connection with LM resistance and to investigate their potential function. We selected 200 chickpea genotypes chosen using FIGS [34]. Over three years (2014, 2015, and 2016), these genotypes were evaluated for LM resistance. The marker-trait association analysis revealed that the H1C092 marker had a significant association with the LM phenotype over two years, whereas the other markers were only associated with one (Table 4). *In silico* PCR analysis was used to determine the possible location of the PCR marker on the chickpea genome and nearby genes (Figure 5). The *in silico* PCR indicated that the H1C092 marker was found on Chr3, and it corresponds to a region located near a gene belonging to the glutathione S-transferase (GST) gene family (Figure 5).

## Discussion

Our findings demonstrated that genotypes with simple leaf types were the most vulnerable to LM damage (LIS = 69), whereas those with multi-pinnate and tiny leaves were resistant (LIS = 23) and moderately resistant (LIS = 4) (Fig. 3). Similarly, Toker *et al.*[39], have screened 15 chickpea lines with three leave types: normal, simple, and multi-pinnate. They found that the plants with simple leave type and large leaflet were susceptible, and the resistant plants had multi-pinnate leave type and small size. El Bouhssini *et al.* [10], found that the number of eggs laid in the susceptible chickpea line ILC3397 was higher than the resistant lines (ILC5901) that had multi-pinnate leave type. Singh and Weigand [40] released three leaf genotypes that are resistant to leaf minor with multi-pinnate and small leaflets (ILC 5901, ILC 7738, and ILC 3800).

The results showed that only a few (35 plants) of F2 plants were resistant; similarly, out of 350 F2 plants, only 35 (10%) showed resistance (LIS =<3), 52 plants (14.9%) were moderately resistant (LIS=4), 121 plants (34.6%) were moderately susceptible (LIS =5, 6), and 142 plants (40.7%) showed susceptible (LIS =7-9). In another study, 174 chickpea germplasm samples were evaluated in the field at three different locations in Pakistan (NARC, AARI, and NIAB). However, resistance to LM was observed in 28 NARC lines, 24 AARI lines, and 30 NIAB lines. Similarly, 44, 47, and 30 lines at NARC, AARI, and NIAB exhibited moderately resistant reactions to LM, respectively [41].

Toker *et al.,* [39] also stated that LM resistance was strongly related to leaf type and leaflet size, but that there was no significant relationship between resistance and pigmentation. Leafminor resistance was found to have a negative relationship with leaf type as well as leaflet size (R = -0. 92; P 0.001); these findings are consistent with Sithanantham and Reed's [42] observation that LM prefers large leaflet chickpea lines. Taleei *et al.,* [43] reported a negative association among leaf size and blight score, implying that genotypes with large leaves may be more susceptible to Ascochyta blight.

The chi-square test revealed that 24 markers (47%) differed from the expected Mendelian ratio of 1:2:1, which could be attributed to gametic [44], zygotic [45], or both selection, chromosomal recombinations with little effect on fertility and chromosome combining throughout meiosis [46], or a correlation to a lethal allele in sperm and eggs phases [47; 48].

A clustering of deformed loci has frequently been discovered inside LGs constructed by different species [49]. Furthermore, a similar clustering pattern of markers was originally described in field peas and chickpeas [50; 51]. Although segregation deformation can be seen in intraspecific crosses, the inclusion of the Lens culinaris ssp. orientalis accession in the hybrid process may have increased the likelihood of lower recombination in the mapping population, therefore segregation distortion. Durán *et al.* [52] employed 17 RAPDs, 13 ISSRs, 41 AFLPs, and one SSR that deviated from Mendelian segregation and found that 24.5% of the markers were distorted in F2 populations.

Results from multiple studies illustrate that segregation distortion can be quite high, particularly in *Cicer* sp. and lentil. Winter *et al.* [51] and Collard *et al.* [20] both reported values of 38.4%, and 14% respectively in these populations. Similarly, Sherman [53] and Xu [54] reported distortion levels of 50% and 25% in chickpea and wheat, respectively. Although the influence of distorted markers is often overlooked, this study found a significant segregation distortion of 38.4% for RILs from a crossover in *Cicer* sp. and 14% in an F2 lentil population [51; 55].

Our findings have revealed five potential QTLs associated with chickpea resistance to leaf miner infestation. These locations are believed to be implicated in several types of resistance mechanisms, including non-preference or antixenosis (the shape of leaves, either normal or multipinnate) and antibiosis (the exudation of oxalic acid from trichomes of resistant and moderately resistant chickpea plants, which reduces injury from infestation). A study by Rector *et al.* [56] made use of 128 AFLP markers, distributed across 30 linkage groups, to identify QTLs correlating to Antixenosis and Antibiosis Mechanisms for *Helicoverpa zea*. Another study identified a major QTL related to antibiosis mechanisms for *Helicoverpa zea*, with a high explanation percentage [57]. The QTL related to maysin production (a glycosyl flavone that controls antibiosis in *H. zea* larvae) explains 55% of the variance in maysin synthesis [58], while the other explains 64%

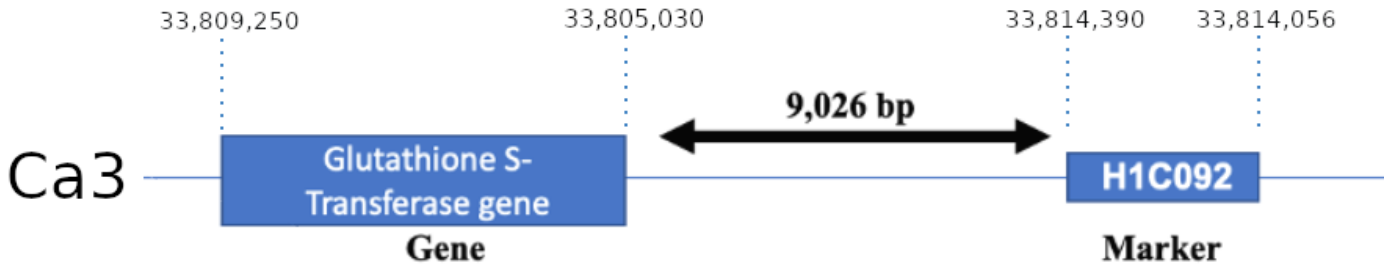


**Figure 5.** Physical location of leaf miner associated marker of H1C092 and its nearby gene.

of the variance in apimaysin synthesis [59].

The potential QTLs were validated using a broad sample of chickpea genotypes over a three-year period to deepen our understanding of their association with LM resistance. Results from the validation showed that not all QTLs showed significant association with LM, but this does not necessarily imply a weak correlation and further studies may be needed. However, one marker was found to have a significant association with LM located on Chr3 near a gene belonging to the glutathione S-transferase (GST) gene family (as shown in Table 4 and Figure 5). GSTs are widely distributed, multi-functional enzymes that play a crucial role in a plant's response to various stress conditions, including biotic stress [60]. GSTs with glutathione peroxidase activity can play an important role in plant antioxidant defense by preventing the spread of hypersensitive response-associated apoptosis [61]. Several studies have shown increased GST enzyme activities in plant-pathogen interactions, and functional studies have revealed that these enzymes can influence antimicrobial resistance in the host plant [62]. Additionally, GST activity has been associated with LM resistance in several plant species including tomato [63] and common bean [64], highlighting its significance as a key for plant resistance.

## Conclusion

Improving breeding programmes to strengthen resources against current and future plant pathogen impacts is crucial. This is the first report for SSR markers associated with LM resistance in chickpea, which may help in guiding and enhancing the use of marker assisted selection to speed up the conventional breeding of LM resistance in chickpea. We validated the potential application of several QTLs linked to LM resistance. Field and experimental validation were extremely useful in confirming the utility of some of these markers. Furthermore, we employed genome annotation to determine the physical location of significant SSR markers and understand their relationship with the chickpea biological resistance. We discovered a putative link between the GST gene family and the resistance to chickpea leaf miner. Our findings suggest that these markers can be used to identify elite chickpea genotypes, and we encourage future researchers to investigate the molecular mechanism of this gene family in chickpea resistance in greater detail.

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